T cell regulation in microgravity - the current knowledge from in vitro experiments con-ducted in space, parabolic flights and ground-based facilities

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DOI: https://doi.org/10.1016/j.actaastro.2014.05.019

Acta Astronautica 104 (2014) 365–377

Contents lists available at ScienceDirect

Acta Astronautica

journal homepage: www.elsevier.com/locate/actaastro

T cell regulation in microgravity – The current knowledge from *in vitro* experiments conducted in space, parabolic flights and ground-based facilities

Swantje Hauschild [a,b,d], Svantje Tauber [a,b,d], Beatrice Lauber [a], Cora S. Thiel [a,b,d], Liliana E. Layer [a], Oliver Ullrich [a,b,c,d,*]

[a] *Institute of Anatomy, Faculty of Medicine, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland*
[b] *Department of Machine Design, Engineering Design and Product Development, Institute of Mechanical Engineering, Otto-von-Guericke-University Magdeburg, Universitätsplatz 2, D-39106 Magdeburg, Germany*
[c] *Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland*
[d] *Study Group "Magdeburger Arbeitsgemeinschaft für Forschung unter Raumfahrt- und Schwerelosigkeitsbedingungen" (MARS), Otto-von-Guericke-University Magdeburg, Universitätsplatz 2, D- 39106 Magdeburg, Germany*

ARTICLE INFO

Article history:
Received 8 March 2014
Received in revised form
12 May 2014
Accepted 15 May 2014
Available online 2 June 2014

Keywords:
Lymphocytes
Adaptive immunity
Spaceflight
Signaling
Gravitational biology

ABSTRACT

Dating back to the Apollo and Skylab missions, it has been reported that astronauts suffered from bacterial and viral infections during space flight or after returning to Earth. Blood analyses revealed strongly reduced capability of human lymphocytes to become active upon mitogenic stimulation. Since then, a large number of *in vitro* studies on human immune cells have been conducted in space, in parabolic flights, and in ground-based facilities. It became obvious that microgravity affects cell morphology and important cellular functions. Observed changes include cell proliferation, the cytoskeleton, signal transduction and gene expression. This review gives an overview of the current knowledge of T cell regulation under altered gravity conditions obtained by *in vitro* studies with special emphasis on the cell culture conditions used. We propose that future *in vitro* experiments should follow rigorous standardized cell culture conditions, which allows better comparison of the results obtained in different flight- and ground-based experiment platforms.

Abbreviations: 5-LOX, 5-lipoxygenase; AP-1, activator protein 1; APO, apoptosis antigen; BL, baseline; ConA, Concanavalin A; CREB, cAMP response element-binding protein; DLR, Deutsches Zentrums für Luft- und Raumfahrt, engl. German Aerospace Center; ESA, European Space Agency; FBS, fetal bovine serum; GCCP, good cell culture practice; GC, ground control; H/W, hardware; HARV, high-aspect ratio vessel; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HS, human serum; hyp-g, hypergravity; IFN, interferon; IL, interleukin; ISS, international space station; LAT, linker of activated T cells; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappaB; PARP, poly (ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cells; PDB, phorbol dibutyrate; PHA, phytohemagglutinin; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; RPM, random positioning machine; RPMI-1640, Roswell Park Memorial Institute-1640 medium; RWV, rotating wall vessel; STAT, signal transducers and activators of transcription; STS, space transportation system; TCR, T cell receptor; TNF, tumor necrosis factor

* Corresponding author at: Institute of Anatomy, Faculty of Medicine, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. Tel.: +41 44 63 55310, 49 391 67 58522; fax: +41 44 63 55498, 49 391 67 12595.
E-mail addresses: oliver.ullrich@uzh.ch, oliver.ullrich@ovgu.de (O. Ullrich).

http://dx.doi.org/10.1016/j.actaastro.2014.05.019
0094-5765/

1. Introduction

Since gravity has been a constant factor throughout the evolution of life, it has shaped the architecture of all biological systems on the Earth. Therefore, one would not be surprised if sudden changes of the gravitational force lead to deviations of normal functions of life. The question of how cellular and molecular functions adapted and therefore depend on the Earth's gravity is of enormous interest. At current levels of knowledge, microgravity leads to a variety of deconditioning symptoms like bone demineralization, muscle atrophy, reduced performance of the cardiovascular system, altered neurovestibular perception as well as strong impairment of the immune system [1]. For the first time, immune dysregulation under space conditions was observed in the 1960s and 1970s. Fifteen of the 29 Apollo astronauts had bacterial or viral infections that occurred during space flight or soon after returning to the Earth. Also latent viruses such as the *varicella zoster* virus were reactivated [2,3]. Subsequent analyses of blood samples of nine astronauts after returning from the Skylab space station revealed that the activation upon mitogenic stimulation of lymphocytes was reduced in comparison with the situation before the mission [4].

Functional disturbance of lymphocytes and, consequently, immune deficiency are discussed as a credible risk for manned long-term space flights [5]. Space flight induced changes in immune response could lead to altered resistance to infections or cancer or to altered hypersensitivity reactions which further yield severe clinical manifestations [6–8]. Therefore, it is of great importance to identify the exact causes and mechanisms of the microgravity-induced human immune system depression. Besides the factor microgravity also extraordinary psychological stress in a confined environment and high radiation levels represent a major and direct “stress factor” at the cellular level. However, since many studies could show that microgravity strongly affects immune cell function [9–11], this factor is currently considered as a major reason for dysregulation of immune cell function during space flight.

During the last three decades, *in vitro* studies with isolated lymphocytes in real and simulated microgravity confirmed effects of reduced gravity at the cellular level. These studies also provided evidence that alterations in molecular mechanisms and signal transduction processes are a direct result of altered gravity. Therefore, isolated lymphocytes represent a superior biological model system to investigate if and how the Earth's gravity is important for cellular and molecular processes inside mammalian cells to function properly. Experiments were performed during manned space flights, on board of orbital, suborbital (sounding rockets) and parabolic flights, and were supported by studies on ground-based facilities aiming to achieve simulated microgravity condition [12]. Since the number and type of experiments during space flights are limited, several ground-based facilities for simulation of microgravity have been developed. These include the fast rotating clinostat, rotating wall vessel (RWV) bioreactor, random positioning machine (RPM), and high-aspect ratio vessel (HARV). Today, most simulation experiments with immune cells have been conducted in the clinostat or the RWV. As they appear to give comparable results to experiments conducted in real microgravity, RWVs and clinostats are recognized as valuable tools for the simulation of microgravity in suspension cell cultures [13].

These experiments under real as well as under simulated microgravity conditions revealed gravity-sensitive functions of non-activated and activated T lymphocytes, which includes cell cycle regulation [14], epigenetic [15] and chromatin regulation [16], differential gene expression [14,17] and microRNA expression profile [18], cell motility [19,20], and regulation of apoptosis [21–23]. Furthermore, expression of cytokines such as interleukin-(IL-2), and interferon-gamma (IFNγ) are changed in microgravity [24].

This review provides an overview of the results obtained over the last 30 years of *in vitro* experiments performed in space and in ground-based simulations with special emphasis on the cell culture conditions used. These results contribute to our current knowledge of how gravitational changes affect human T lymphocytes *in vitro*.

2. T cell regulation in real and simulated microgravity experiments *in vitro*

2.1. Reduced T cell activation

Up to now, several studies with isolated T lymphocytes were carried out in real (Table 1) and simulated (Table 2) microgravity. For the first time, *in vitro* activation of T lymphocytes isolated from human peripheral blood under microgravity conditions was conducted in space during the Spacelab 1 mission aboard the space shuttle Columbia (Space transportation system 9, STS-9) in 1983. The reactivity of peripheral blood lymphocytes to stimulation with the mitogen Concanavalin A (ConA) was almost completely lost [25,26]. These results were confirmed in two subsequent experiments performed in the BIORACK facility of Spacelab D-1 aboard the space shuttle Challenger (STS-61-A) in 1985 [27–30] which included 1*g* in-flight reference centrifuge controls. *in vitro* activation of human lymphocytes was significantly reduced by almost 100% in microgravity compared with control cells on an 1*g* onboard reference centrifuge [27–30]. This reduced activation was confirmed in the ground-based facilities RWV, clinostat and RPM [12,31,32].

The consideration that binding of the mitogen ConA is altered in microgravity, was examined in experiments performed on four sounding rockets providing 7 min resp. 13 min microgravity [33,34]. Using fluorescent-labelled ConA, these experiments showed that binding of the mitogen to the membrane was principally not affected; only a slight delay of patching and capping was observed.

2.2. Influence of cell-to-cell and cell–substrate interactions on T cell function

In real and simulated microgravity, the lack of sedimentation may lead to reduced cell-to-cell and cell–substrate interactions, which in turn could contribute to the reduced proliferative response to mitogenic stimuli observed in altered gravity. However, phytohemagglutinin (PHA) stimulation of peripheral blood mononuclear cells

Table 1
Summary of experiments performed under real microgravity conditions, (references are in chronological order of year of publication).

Reference	Cell type	Research platform	Activation	Exposure/ Activation time	Gravity conditions	Cell culture medium	Results
[25]	PBMCs	Spacelab 1; STS-9	ConA	71 h	0g 1g GC	RPMI-1640, 20% HS, 40 mM HEPES, 5 mM $NaHCO_3$, gentamycin	Mitogenic activation of human lymphocytes in microgravity was less than 3% of the ground control.
[29]	Whole blood culture of astronauts	Spacelab D1; STS-61-A	ConA	78 h	L-9, L-2, L+3, R+0, R+7, R+13 1g in-flight	Peripheral blood diluted 1:10 (v/v) in: RPMI-1640, 20% HS, 40 mM HEPES, 5 mM NaHCO3, heparin, gentamycin	At 1g, the activation of lymphocytes during and after the flight was attenuated compared to the pre-flight values before the flight. Under 0g conditions lymphocytes were not activated compared to 1g in-flight control.
[30]	PBMCs	Spacelab D1; STS-61-A	ConA	36, 48, 72+96 h	0g 1g in-flight	RPMI-1640, 20% HS, 40 mM HEPES, 5 mM $NaHCO_3$, heparin, gentamycin	*in vitro* activation of human lymphocytes by the mitogen ConA was reduced by 90% in microgravity.
[37]	Jurkat cells or co-culture of Jurkat+THP-1 cells	Biosatellite Cosmos 2044 (BION 9)	PMA/A23187 calcimycin, or anti-CD3 mAb in the presence of THP-1 cells	24 h	0g 1g GC	RPMI-1640, 10% FBS, 25 mM HEPES, 12 mM $NaHCO_3$, 1 mM sodium pyruvate, 2 mM glutamine	IL-2 secretion was suppressed in PMA/Calcimycin activated Jurkat cells in microgravity. Cell-to-cell contacts took place in microgravity, leading to normal production of IL-1 and IL-2 compared to ground controls.
[43]	PBMCs	STS-43	ConA	24, 48 h	0g 1g GC	RPMI-1640, 10% FBS, 28 mM HEPES	Lymphocytes in space secreted much more IFNγ than on the ground.
[35]	PBMCs	Spacelab SLS-1; STS-40	ConA or ConA/ Cytodex beads	72 h	0g 1g in-flight 1g GC	RPMI-1640, 20% FBS, 40 mM HEPES, 5 mM $NaHCO_3$, 4 mM glutamine, gentamycin	Activation in microgravity was almost doubled on cytodex beads. IFNγ production was increased by 300% on cytodex beads.
[36]	PBMCs	Spacelab SLS-1; STS-40	ConA or ConA/ Cytodex beads	52+71 h	0g 1g in-flight 1g GC	RPMI-1640, 20% FBS, 40 mM HEPES, 5 mM $NaHCO_3$, gentamycin	ConA-activation in microgravity was almost doubled on cytodex beads. Also IL-2 and IFNγ production was strongly increased and IL-2R expression was in the normal range with cytodex beads in microgravity.
[39]	PBMCs	Spacelab IML-2; STS-65	ConA	72 h	0g 1g in-flight 1g GC 1.4g ground	RPMI-1640, 10% FBS, 40 mM HEPES, 5 mM $NaHCO_3$, 4 mM glutamine, gentamycin	Addition of exogenous IL-1+IL-2 was not capable of preventing the loss of activity at 0g, but it restored slightly but significantly the production of IFNγ at 0g.
[38]	PBMCs	Spacelab IML-2; STS-65	ConA	46+78 h	0g 1g GC	RPMI-1640, 10% FBS, 40 mM HEPES, 5 mM $NaHCO_3$, 4 mM glutamine, gentamycin	Aggregate forming of ConA activated cells at 0g as well as in 1g indicated that Cell-cell contacts occur in microgravity. At 0g the mean velocity of free cells did not decrease with incubation time indicating that cell cycle progression is inhibited.
[51]	Jurkat cells	Spacelab IML-2; STS-65	A23187 calcimycin	1 h	0g 1g in-flight 1g GC 1.4g ground	RPMI-1640, 10% FBS, 2 mM glutamine, penicillin-streptomycin-neomycin	Relative distribution of PKC in the cytosolic and nuclear fractions varied as a function of the g level, with cytosolic PKC increasing with increasing g level, whereas nuclear PKC decreased.
[33]	PBMCs Jurkat cells	sounding rocket MASER-3, MASER-4, MAXUS-1B	ConA	7 min (MASER) 12.5 min (MAXUS)	0g 1g GC hyp-g (13g)	RPMI-1640, 10% FBS, 20 mM HEPES, 5 mM $NaHCO_3$, 1 mM sodium pyruvate, 4 mM glutamine, gentamycin	Binding of ConA to membrane was not affected by microgravity, whereas patching and probably capping were slightly retarded. Structural changes of intermediate filaments of vimentin as well as of the microtubule network occurred. Cell motility and cell-cell-contacts occurred in microgravity.
[21]	Jurkat cells	STS-76	non-stimulated	4, 24, 48 h	0g 1g in-flight 1g GC 1.4g ground	RPMI-1640, 10% FBS, 12.5 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, penicillin-streptomycin	Significant changes in the microtubule cytoskeleton. Filaments were shortened, coalesced, lacked normal branching at the cell membrane, and MTOCs were disrupted. Time-dependent increase in the apoptosis-related factor, Fas/ APO-1 in culture medium of flown cells (0g+1g).
[32]	PBMCs, purified T cells	STS-81 and STS-84	Okt3/CD28 beads or PDB/Ionomycin or Leu4 or Leu4 beads	24 h	0g 1g in-flight	RPMI-1640, 10% FBS, 2 mM glutamine, penicillin-streptomycin	Suppressed CD25 and CD69 surface expression after activation.

Table 1 (*continued*)

Reference	Cell type	Research platform	Activation	Exposure/ Activation time	Gravity conditions	Cell culture medium	Results
[34]	Jurkat cells	sounding rocket MAXUS-2	ConA	12 min	0g 1g in-flight 1g GC	RPMI-1640, 10% FBS, 20 mM HEPES, 5 mM $NaHCO_3$, 1 mM sodium pyruvate, 4 mM glutamine, gentamycin	Binding of ConA to Jurkat cells was not influenced by microgravity, whereas patching of the ConA receptors was slightly retarded. Structural changes of vimentin in microgravity. Appearance of large bundles significantly increased in the microgravity samples. No changes were found in the structure of actin and in the co-localization of actin in the inner side of the cell membrane with ConA receptors after binding of the mitogen.
[44]	PBMCs	4 separate space shuttle missions	PMA/Ionomycin or PMA or PHA	5 h or 24 h	L-10, R+0, R+3	RPMI-1640, 10% FBS, penicillin-streptomycin,	CD4 and the CD8 T cell subsets exhibited a reduced ability to produce IL-2 following space flight. IFNγ production was significantly reduced in the CD4+T cell subset, and production of IFNγ in the CD8+ T cell subset was unchanged.
[22]	Jurkat cells	STS-80 and STS-95	non-stimulated	STS-80: 0, 4, 24, 48, 75 h STS-95: 4, 24, 48 h	0g 1g in-flight 1g GC 1.4g ground	RPMI-1640, 10% FBS, 12.5 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, nonessential amino acids penicillin-streptomycin	Apoptosis increased during space flight and the release of sFas was time-dependent and microgravity-related.
[58]	Jurkat cells	STS-95	non-stimulated	4, 24, 48 h	0g 1g GC ground vibration samples	RPMI-1640, 10% FBS	Gene expression of 11 cytoskeleton-related genes was up-regulated in space-flown cells. Microgravity led to differential expression of genes that regulate growth, metabolism, signal transduction, adhesion, transcription, apoptosis, and tumor suppression.
[47]	Jurkat cells	space flight	non-stimulated	4, 48 h	0g 1g in-flight 1g GC 1.4g ground	RPMI-1640, 10% FBS, 12.5 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, nonessential amino acids penicillin-streptomycin	Mitochondria clustering and morphological alterations of mitochondrial cristae were observed. Jurkat cells underwent cell divisions during space flight but also a large number of apoptotic cells was observed. Mitochondria were unevenly distributed in cells which might be a result of the seen microtubule disruption.
[50]	primary T cells	STS-81	PDBu/ Ionomycin or CD3 beads	0, 10, 60 min	0g 1g in-flight 1g GC 1.4g ground	RPMI-1640, 10% FBS, 25 mM HEPES, 12 mM $NaHCO_3$, 1 mM sodium pyruvate, 2 mM glutamine, penicillin-streptomycin-neomycin	Microgravity reduced the translocation of PKCδ to particular cell fractions. PKCβII translocation was not significantly different between 0g and 1g.
[45]	Whole blood culture of astronauts	STS-114, STS-121, STS-115 and STS-116 ISS Expeditions 11–14	CD3/CD28	short- and long-duration space flight	L-180, L-65, L-10, R+0, R+3, R+14, R+30	Peripheral blood diluted 1:10 (v/v) in RPMI-1640	For Shuttle crewmembers, early T cell activation was elevated post-flight; however, the percentage of T cell subsets capable of being stimulated to produce IL-2 and IFNγ was decreased. For ISS crewmembers, early T cell activation was reduced immediately post-flight. The percentage of T cells capable of producing IL-2 was reduced, but IFNγ percentages were unchanged.
[16]	Jurkat cells	8^{th} DLR Parabolic Flight Campaign	PMA or CD3/CD28	20 s	0g 1g GC	RPMI-1640, 10% FBS, penicillin-streptomycin	p53 phosphorylation increased in non-activated as well as in CD3/CD28-activated Jurkat cells after 20 s microgravity. Also MEK phosphorylation was enhanced in PMA and in CD3/CD28 activated cells microgravity compared to 1g in-flight controls.
[14]	Jurkat cells, CD4+ T lymphocytes	9^{th} 10^{th} and 13^{th} DLR and 45^{th} ESA Parabolic Flight Campaign	PMA or CD3/CD28	20 s	0g 1g in-flight	RPMI-1640, 10% FBS, penicillin-streptomycin	p21 Waf1/Cip1 mRNA expression was enhanced after 20 s microgravity. This expression was dependent from histone acetylation. Also Tyr15-phosphorylation of cdc2 was enhanced.
[23]	PBMCs	BIO-4 mission, ISS Expedition 18	ConA	0, 3, 24, and 48 h	0g 1g in-flight 1g GC	RPMI-1640, 10% FBS, 40 mM HEPES, 300 μg/ml glutamine, gentamycin	Exposure to microgravity increased DNA fragmentation and PARP protein expression, as well as mRNA levels of p53 and

							calpain; these changes were paralleled by an early increase of 5-LOX activity.
[60]	primary CD4+ T cells	sounding rocket MASER-12	ConA/CD28	6 min	0g 1g in-flight hyp-g 1g GC	RPMI-1640	Hypergravity led to dysregulation of several key signal proteins involved in early TCR signaling. These key proteins were not severely disturbed in the following microgravity phase.

1g GC=1g ground control; 5-LOX=5-lipoxygenase; APO=apoptosis antigen; ConA=Concanavalin A; FBS=fetal bovine serum; HEPES=4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HS=human serum; hyp-g=hypergravity; IL=interleukin; IFN=interferon;; ISS=international space station; PBMC=peripheral blood mononuclear cells; PDB=phorbol dibutyrate; PHA=phytohemagglutinin; PMA=phorbol-12-myristate-13-acetate; PKC=protein kinase C; PARP=poly (ADP-ribose) polymerase; RPMI-1640=Roswell Park Memorial Institute-1640 medium; STS=space transportation system; TCR=T cell receptor; TNF=tumor necrosis factor; n/a=not available.

(PBMCs) cultured in Teflon bags, which reduce cell–substratum interactions, did not significantly affect proliferation compared to cultures in standard cell culture flasks [31]. Other experiments aboard STS-40 where PBMCs were immobilized on microcarrier beads prior to ConA stimulation showed that lymphocyte activation was almost doubled [35,36]. Proliferation of T lymphocytes was totally inhibited in an RWV experiment where cells were stimulated with CD2/CD28 and CD3/CD28. These stimuli activate cells without requiring co-stimulatory signals by cell-to-cell interaction [31]. Furthermore, experiments with human PBMCs and/or Jurkat T cells performed aboard the biosatellite BION-9, the space shuttle STS-65 and aboard three sounding rockets (MASER-3, -4, and MAXUS-1B) could show that cellular interactions occur in microgravity, since aggregates of lymphocytes were observed [33,37,38]. Therefore, it is more likely that alterations in signal transduction rather than absence of cell-to-cell interactions are responsible for depression of T cell function.

2.3. Microgravity changes the patterns of cytokine release

IL-2 and IL-2 receptor (IL-2 R) interaction plays a critical role as a required co-stimulatory signal for full T cell activation. Thus, the reduced ability of T cells to proliferate and differentiate into functional effector cells upon activation in microgravity [9,25] could also be caused by alterations in IL-2 secretion or IL-2 R surface expression, resulting in an impairment of the positive regulatory feedback loop. Experiments performed with human PBMCs during several space flights (STS-40, STS-60,STS-81 and STS-84) revealed that IL-2 secretion as well as the level of IL-2 R expression are strongly reduced in microgravity [32,36,39]. Moreover, experiments with PBMCs or primary human T cells in simulated microgravity provided by clinostat, RWV, or RPM could confirm these results [31,32,40] and could further show that the genetic expression of IL-2 and its receptor was inhibited upon activation [41,42]. However, co-stimulation with sub-mitogenic concentrations of phorbol-12-myristate-13-acetate (PMA) could restore proliferative response and surface expression of IL-2 R [31,32].

Analyses of IFNγ secretion upon mitogenic stimulation of cells flown in space and cells from astronauts flown on several space shuttle missions revealed that in-flight stimulation of PBMCs with ConA led to increased IFNγ release in comparison to ground controls [43], whereas PMA/Ionomycin stimulation of samples which were taken directly after landing from astronauts exhibited significant reduction of IFNγ secretion by CD4+ T lymphocytes and unchanged secretion by CD8+ T lymphocytes [44]. Furthermore, comparison of whole blood culture samples collected from astronauts of short- (space shuttle) and long-duration (ISS) flights showed that after the short-duration flight the percentage of T cells producing IFNγ was decreased, whereas after the long-duration flight T cell percentage producing IFNγ was unchanged [45]. Exposure of lymphocytes to simulated microgravity in an RWV led to initial suppression of IFNγ secretion, which was restored to normal levels after three days [31].

Table 2
Summary of experiments performed under simulated microgravity conditions using ground-based model systems, (references are in chronological order of year of publication).

Reference	Cell type	Research platform	Activation	Exposure/ Activation time	Gravity conditions	Cell culture medium	Results
[19]	PBMCs	RWV	CD3/IL-2	24 h to 9 d	1g sim. 0g	RPMI-1640 10% FBS penicillin-streptomycin	Locomotion into collagen type I matrix was inhibited.
[31]	PBMCs, primary T cells	RWV	PHA	24–72 h	1g sim. 0g	RPMI-1640 10% FBS penicillin-streptomycin	PHA stimulated PBMCs showed suppressed proliferation and reduced IL-2 secretion. Secretion of IFNγ was initially suppressed but returned to normal levels after 3d. CD25 and CD69 surface expression was reduced by over 50%. Exogenous IL-2 didn't restore microgravity effect on PHA stimulated PBMCs. Restoring of proliferation ability in PHA stimulated cultures using sub-mitogenic concentrated PMA.
[41]	PBMCs	Clinostat, RPM	ConA	1–12 h	1g sim. 0g	RPMI-1640, 10% FBS, 50 mM HEPES, 5 mM NaHCO3, gentamycin	Clinostat and RPM experiments showed a decrease in IL-2 and IL-2R gene expression.
[32]	PBMCs, primary T cells	2D-clinostat	PHA or Leu4	3, 24, and 48 h	1g sim. 0g	RPMI-1640, 10% FBS, 2 mM glutamine, penicillin-streptomycin	Reduced proliferation of PHA or Leu4 stimulated PBMCs after 48 h clinorotation. Leu4 stimulated PBMCs showed suppressed expression of CD69 and CD25 activation marker after 24 h clinorotation and also cell cycle progression was inhibited. TCR internalization occurred slower under simulated microgravity condition. PMA co-stimulation of activated PBMCs could restore surface expression of CD69 and CD25.
[12]	PBMCs	RPM	ConA	72 h	1g sim. 0g	RPMI-1640, 10% FBS, 40 mM HEPES, 2 mg/mL NaHCO3, gentamycin	Suppressed proliferation of ConA activated PBMCs of the same order of magnitude as in space after 72 h exposure to RPM.
[71]	PBMCs	RWV	IL-2	2–8d	1g sim. 0g	AIM-V (chemically defined serum-free medium)	Exogenous IL-2 contributed to maintenance of cell viability but could not induce CD25 surface expression or restore cytokine (IFNγ, IL-1β, and TNFγ) secretion totally.
[54]	PBMCs, preliminary activated primary T cells	RWV	CD3/IL-2	2, 4, 6, 18 and 24 h	1g sim. 0g	RPMI-1640, 10% FBS	Inhibition of radiation- and activation induced programmed cell death. Microgravity might influence interaction of membrane-bound Fas and FasL on the cell surface. Inhibition of PCD in activated T lymphocytes in microgravity may not be related to changes in expression of Bcl-2 or Bax antigen.
[20]	PBMCs	RWV	PMA	24, 48, 72, 96 h	1g sim. 0g	RPMI-1640, 10% FBS	Locomotion of non-stimulated PBMCs was inhibited after 24 h of RWV culture with extent of locomotion loss at 72 h. Addition of PMA to PBMCs under simulated microgravity conditions restored locomotory function.
[52]	primary T cells	RPM	ConA/ CD28+Protein *G*	6, 15, 30 min	1g sim. 0g	RPMI-1640, 10% FBS, 40 mM HEPES, 5 mM NaHCO3, 4 mM glutamine gentamycin	Exposure to simulated microgravity of activated T cells led to altered PKC isoform distribution in the three fractions nucleus, cytosol and plasma membrane.
[53]	PBMCs	RWV	non-stimulated	24, 48, 72, 96 h	1g sim. 0g	RPMI-1640, 10% FBS	Exposure to RWV led to a decrease in the expression of specific calcium-independent PKC isoforms in PBMCs at both the RNA and protein levels. RWV cultured PBMCs also showed a 56% decrease in phosphorylated PLC-γ1.
[40]	primary T cells	Clinostat	CD69/PMA	48 h	1g sim. 0g	RPMI-1640, 10% FBS, 40 mM HEPES, 5 mM NaHCO3, 2 mM glutamine, Penicillin-Streptomycin	Significant higher calcium concentration was detectable in clinorotating CD69/PMA stimulated cultures than in control cells. Mitochondrial membrane hyperpolarization due to activation was more prolonged since after 20 h and was followed by depolarization in a fraction of cell population. Clinorotation significantly inhibited IL-2 secretion and also proliferation was impaired in CD69/PMA stimulated T cells.
[42]	primary T cells	RPM	ConA/CD28	30 min	1g sim. 0g	n/a	99 genes were significantly up-regulated during early T cell activation in normal gravity. Under simulated microgravity conditions the majority of those genes showed no significant mitogen induced gene expression. 28% of these genes were component of NF-κB signaling or had evidence for regulation by NF-κB.

							PKA signaling might be affected since activation of CREB by phosphorylation was significantly blocked by simulated microgravity.
[72]	PBMCs Lymphoblastoid cell lines LB and COR3	RPM	ConA	8 or 24 h	1*g* sim. 0*g*	DMEM, 10% FBS, 25 mM HEPES, 2 mM glutamine, penicillin-streptomycin	Apoptosis induction, release of sFas and fluctuation of PARP activity occur transiently and only to a minor extent. Decrease in intracellular concentration of ATP. Microgravity exposure might induce a condition of metabolic 'quiescence'.
[55]	Preliminary activated T cells	RWV	CD3/IL-2	24 h	1*g* sim. 0*g*	RPMI-1640, 10% FBS, 100 U/mL IL-2	Gene chip microarray analysis revealed microgravity-induced changes in the expression of genes belonging to functional categories immune response, cell proliferation and differentiation, protein folding, transport and degradation, as well as apoptosis.
[73]	PBMCs	Clinostat	CD3/CD28 beads	2.5–16 h	1*g* sim. 0*g*	RPMI-1640, 10% FBS, 5 ng/mL PMA, 200 ng/mL Ionomycin	Clinorotation differentially affects signaling pathways in T lymphocytes; the Ca++/Cn signaling remains active, whereas PKC pathway might be inhibited since activation of fos and NF-κB is inhibited.
[56]	PBMCs	RWV	non-stimulated	24, 72 h	1*g* sim. 0*g*	n/a	Culturing non-stimulated PBMCs in RWV led to differential gene expression: -Down-regulation of T cell activation genes DAG kinase, Ser/Thr kinase and Tyr kinase–Up-regulation of HSPA1A (e.g. HSP-70) and down-regulation of HSP9A9B (i.e., HSP-90)–Up-regulation of angiogenic factor PIGf
[59]	PBMCs	RWV	PHA or PHA+PMA	48 h	1*g* sim. 0*g*	RPMI-1640, 10% FBS, 2 mM glutamine, Penicillin-Streptomycin	PMA co-stimulation of primary human T cells cultured in the RWV restored PHA-induced activation of the CD8+ and CD4+ T cell subsets as well as naïve and memory CD4+ T cells.
[74]	PBMCs	Clinostat	PHA	24–48 h	1*g* sim. 0*g*	RPMI-1640, 10% FBS, 1% HEPES, 1% glutamine, gentamycin, fungizone	Decrease in proliferation and viability after 48 h of rotation in the 3-D clinostat.
[57]	Human T cells	RWV	non-stimulated	4 h, 72 h, and 7 days	1*g* sim. 0*g*	DMEM F-12, 10% FBS	Exposure to simulated microgravity condition decreased the expression of DNA repair genes, of cell cycle genes of and anti- and pro-apoptotic genes. Simulated microgravity conditions caused DNA damage.
[75]	primary CD4+ T cells	RWV	CD3/CD28	5–90 min	1*g* sim. 0*g*	RPMI-1640, 10% FBS, 2?mM glutamine, Penicillin-Streptomycin	TCR signaling through DAG remains intact during culture in the RWV. Thus, simulated microgravity seems to prevent T cell activation by modulating the cellular response to the TCR signal rather than by abrogating or limiting the signal itself.
[15]	Human T cells	RWV	non-stimulated	72 h and 7d	1*g* sim. 0*g*	DMEM F-12, 10% FBS	Microgravity induced epigenetic changes in DNA methylation and chromatin histone modifications (decreased expression of DNMT1 and HDAC1).
[16]	Jurkat	Clinostat	PMA or CD3/CD28	5 min	1*g* sim. 0*g*	RPMI-1640, 10% FBS, Penicillin-Streptomycin	Simulated microgravity enhanced phosphorylation of the MAP kinases ERK-1/2, MEK, and *p*38 and inhibited the nuclear translocation of NF-kB, either in non-stimulated or in stimulated cells.
[18]	TK6 human lymphoblastoid cells	RWV	non-stimulated	72 h	1*g* sim. 0*g*	RPMI-1640, 15% FBS, Penicillin-Streptomycin	Simulated microgravity altered miRNA expression which influenced several genes that are involved in the regulation of the NF-κB-related pathway network.
[14]	Jurkat	Clinostat	CD3/CD28 or PMA	5, 10 and 15 min	1*g* sim. 0*g*	RPMI-1640, 10% FBS, Penicillin-Streptomycin	Clinorotated Jurkat cells showed differential protein expression of cell cycle regulatory proteins: enhanced expression of *p*21 Waf1/Cip1 protein, less cdc25C protein expression and enhanced Ser147-phosphorylation of cyclinB1 after CD3/CD28 stimulation
[17]	primary CD4+ T cells	RWV	CD3/CD28 beads	1.5*p*h	1*g* sim. 0*g*	RPMI-1640, 10% FBS	Expression of the immediately early genes cREL, TNF, EGR1, EGR2, and JUNB was significantly reduced in primary CD4+ T cells cultured under RWV conditions.
[76]	PBMCs, primary T cells	RPM	ConA (PBMCs) or ConA/CD28 (T cells)	20–22 h	1*g* sim. 0*g*, 0.2*g*, 0.4*g*, 0.6*g*	RPMI-1640 10% FBS	Lymphocyte activation depends on partial gravity exposure. In simulated 0.2*gg* activated cells were as poorly activated as under simulated 0*gg*, whereas cells activated at simulated 0.6*gg* responded similarly to the 1*g* control. The activation level of the 0.4*g* exposed cells was almost in the top of 0.2*g* and 0.6*g*.

ConA=Concanavalin A; FBS=fetal bovine serum; HARV=high-aspect ratio vessel; HEPES=4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL=interleukin; IFN=interferon;; PBMC=peripheral blood mononuclear cells; PHA=phytohemagglutinin; PMA=phorbol-12-myristate-13-acetate; PKC=protein kinase C; PARP=poly (ADP-ribose) polymerase; RWV=rotating wall vessel; RPM=random positioning machine; RPMI-1640=Roswell Park Memorial Institute-1640 medium; TCR=T cell receptor; TNF=tumor necrosis factor; n/a=not available.

Bead-attached cells also showed markedly different cytokine profiles in comparison to cells in suspension culture: Especially IL-2, IFNγ and tumor necrosis factor-alpha (TNFα) were significantly increased [35,36].

2.4. Microgravity-induced changes in cytoskeletal structures and cell motility

The cytoskeleton is responsible for giving a cell its shape and for generating the forces required for cell motility. It is an internal network of at least three types of cytosolic fibers: actin filaments, microtubules and intermediate filaments. Significant changes in the cytoskeletal structure, which plays an important role not only in cell motility but also in receptor signaling integrity [46], were observed. Structural changes of vimentin filaments, and the microtubule network were reported in several independent experiments in real microgravity [21,33,34,47].

Since lymphocyte migration is fundamental to keep the organism under immunological surveillance and a crucial event in the systemic immune defense, investigating the impact of microgravity on this cellular function is important to understand the immune response in microgravity. Besides cell-to-cell interactions also cell motility is important for cell communication and signal transmission. Observation of cell motility under microgravity conditions revealed that T cells were motile but the motility did not decrease with increasing stimulation time, which indicates that cell cycle progression was inhibited [33,38]. Simulating microgravity by RWV exhibited that locomotion of PBMCs was inhibited after 24 h [19,20] but addition of PMA to RWV culture restored cell motility [20].

2.5. Dysregulated distribution of PKC isoforms

Since the cytoskeleton is involved in signal transduction, microgravity-induced disorganization of cytoskeletal structures could lead to disturbed localization of signaling molecules. Different protein kinase C (PKC) isoforms are associated with several cytoskeletal elements, in particular intermediate filaments and stress fibers [48,49]. Upon T cell activation, the PKC isoforms normally are redistributed to distinct cellular compartments. In two experiments exposing Jurkat T cells and primary human T cells to microgravity during space flight, intracellular translocations of PKC isoforms were investigated. The results showed that the relative distribution of PKC isoforms in particular cell fractions was different from in-flight samples compared to 1g ground controls [50,51]. These results were confirmed in an experiment with primary T cells in an RWV [52]. Furthermore, mRNA as well as protein expression of specific calcium-independent PKC isoforms was inhibited in an RWV PBMC culture [53].

2.6. Increased apoptosis

Apoptosis may also contribute to the decreased proliferative response of lymphocytes in real microgravity. Actually, biochemical and microscopic studies revealed that the rate of apoptosis in Jurkat T lymphocytes was increased in microgravity conditions [21–23], which was reflected in time-dependent release of apoptosis-related factors like Fas/APO1 in the culture medium during exposure of approximately 2 days real microgravity aboard different space shuttle flights [21,22]. Furthermore, microgravity led to increased DNA fragmentation, poly (ADP-ribose) polymerase (PARP) protein expression and p53 and calpain mRNA. These changes were paralleled by an early increase of 5-lipoxygenase (5-LOX) activity [23]. In an experiment that we performed during the 8th DLR (German Aerospace Center) parabolic flight campaign, we could observe an increase of p53 phosphorylation after 20 s real microgravity [16]. Risin and Pellis [54] reported that radiation and activation-induced programmed cell death in T lymphocytes was inhibited under simulated microgravity conditions.

2.7. Differential gene expression in microgravity

The phenomenological characteristics of reduced T cell activation caused by microgravity are well described so far. However, the exact underlying molecular mechanisms are unknown. Therefore, during the last decade, several studies focused on the effect of altered gravity at the level of gene transcription [14,17,42,55–58]. The effect of microgravity on global gene expression was evaluated in cells cultured in real microgravity or in ground based simulations. Microarray analysis revealed that under microgravity conditions (ISS “Astrolab” and RPM) the expression of immediately early genes which are regulated primarily by transcription factors NF-κB, CREB, ELK, AP-1 and STAT were down-regulated relative to 1g controls [17,42]. The overall observed changes in gene expression induced by gravitational changes comprised a number of genes associated with cell stress response [56], cell proliferation and differentiation [42,55,56], cell cycle regulation [14,57], protein folding [55], DNA repair [57], transport and degradation [55], apoptosis [55,57,58], as well as differences in several cytoskeletal genes [58]. These results demonstrated the broad spectrum of gene expression modulations in reduced gravity. Further experiments with primary human T cells revealed that microgravity induced epigenetic changes in DNA methylation and chromatin histone modifications [15]. In numerous experiments that we performed during several parabolic flight campaigns (9th, 10th and 13th DLR and 45th ESA Parabolic Flight Campaign), we could also show that differential mRNA expression induced by microgravity was probably dependent on histone acetylation [14].

Up to date it is not elucidated if and how microgravity affects T cell signaling, in particular the membrane proximal and cytoplasmic signal transduction cascades and the IL-2/IL-2 R activation loop. Although some studies suggested that microgravity affects PKC [50,51], adding PMA to treated cells in simulated microgravity could restore T cell activation [31,59], normal surface receptor expression [32], and cell motility [20]. Moreover, the first activation signals, binding, patching and capping of ConA occurred normally [9]. Thus, the gravisensitive cellular targets were suggested to be located upstream from PKC and downstream from T cell receptor (TCR)/CD3 complex, where the lipid raft-associated signalosome complex is located.

2.8. Influence of microgravity on membrane proximal T cell receptor signaling

Since transcriptional effects of microgravity appeared within 20 s [14], it seemed possible that the signaling cascade triggered by T cell activation, beginning with cell surface receptors, changes within a few minutes. Therefore, in a recent study, we investigated the impact of microgravity on key molecules of the early T cell activation signaling events [60]. Experiments with primary human CD4+ T lymphocytes were conducted under real microgravity conditions on board of the sounding rocket MASER-12. In addition to the microgravity effects on CD4+ T lymphocytes, the influence of the hypergravity phase during the rocket launch (baseline (BL) samples) and of cultivating the cells in the experimental hardware (H/W) were investigated. We tested the influence of gravitational changes on key molecules involved in T cell receptor signaling of resting as well as of ConA/CD28 activated CD4+ T lymphocytes and quantified components of the T cell receptor, membrane proximal signaling events, MAPK signaling, IL-2 R, histone modifications and the cytoskeleton in non-activated as well as in ConA/CD28 activated T lymphocytes. The results are summarized in Table 3.

The hypergravity phase during the launch of MASER-12 resulted in a down-regulation of the both surface receptors IL-2R and CD3 and reduced overall intracellular tyrosine phosphorylation, p44/42-MAPK phosphorylation and histone H3 acetylation, whereas phosphorylation of the linker of activated T cells (LAT) protein was increased. Non-activated T cells showed a reduction of CD3 and IL-2R expression at the cell surface due to microgravity in comparison to the 1*g* H/W ground control. *p*44/42-MAPK-phosphorylation was also reduced after 6 min microgravity compared to the 1*g* H/W ground controls, but also in direct comparison between the in-flight microgravity samples and the 1*g* reference centrifuge control. In contrast, 5 min clinorotation and 20 s real microgravity led to an increase of phosphorylated p44/42 MAPK in non-activated Jurkat T cells as well as in PMA or CD3/CD28-activated Jurkat T cells [16]. In activated T cells, the reduced CD3 and IL-2 receptor expression after the rocket launch of MASER-12 recovered significantly under in-flight 1*g* conditions, but not in microgravity. Beta-tubulin increased significantly during the microgravity phase, but not when cells were re-exposed to 1*g* at the on-board reference centrifuge.

We suggest from this data that microgravity might not severely disturb key proteins of membrane proximal signaling in the first 6 min. Thus, it can be assumed that dysregulation of functional T cell activation occurs downstream of the T cell receptor signaling, such as at the level of gene expression regulation.

3. Conclusion

A large number of studies on T lymphocytes were carried out in microgravity which clearly revealed, that individual cells are sensitive to changes of the gravitational force. The results of these experiments performed in space and in ground-based simulations contributed to the knowledge of how alterations in gravity influence basic cellular mechanisms. The influence of microgravity on T lymphocyte function was reflected in a variety of phenomenological cellular responses that can be grouped into different categories (Table 4) and is summarized in Fig. 1.

Since microgravity simulation provided comparable results to experiments conducted in real microgravity [13], it was possible to carry out a variety of experiments that would not have been possible to this extent only by space experiments. Different incubation times, with a range from 20 s to 8 days, might allow us to identify potential adaptation processes to gravitational changes. However, it was not yet possible to create an overall hypothesis from these various effects and to locate a possible primary mechanism underlying the effects of altered gravity on immune cells.

Considering the experimental conditions of each study summarized in Table 1 and Table 2, it is striking how different these conditions between the individual experiments were been. Stimuli for T cell activation range from mitogens (ConA and PHA), to phorbol ester (PMA and PDB), to calcium ionophores (calcimycin and Ionomycin), up to antibodies against CD3, CD28, CD69, or IL-2 surface receptors. Furthermore, different basal media were used for cultivation lymphocytes ranging from the most widely used RPMI-1640, to DMEM, DMEM F-12, up to the chemically-defined media AIM-V. These used basal media have mostly been supplemented with various additives in different concentrations. In several experiments, for pH buffering, HEPES was used in concentrations reaching from 12.5 mM to 50 mM, sometimes also in combination with 5 mM or 12 mM sodium bicarbonate. Other experiments, however, were conducted without any HEPES or sodium bicarbonate. Some cell culture media contained 1 mM sodium pyruvate and/or 2 mM–4 mM glutamine. Almost all media used for T cell culture in the presented studies were supplemented with fetal bovine serum (FBS). The used concentrations range from 10%, to 15% up to 20%.

As a highly complex mixture, serum provides several factors which stimulate cell proliferation and differentiation, serve as a source of nutrients, bind toxic substances, e.g. free radicals, and support cell adhesion. However, the chemical composition of sera is ill defined and the concentrations of components vary between different batches. These variations influence cell signaling, cell regulation and in turn gene expression.

Therefore, comparability of the several studies that have been conducted to obtain the overall picture and to possibly locate the primary microgravity-induced mechanism is not given. However, diversity of assays and experiments set-ups could represent a chance for new discoveries beyond old paths. This deficiency in standardization has to be regarded inacceptable nowadays, since maintenance of high standards are being introduced into almost every scientific practice in order to maximize reproducibility, reliability, acceptance and successful implementation of results. Guidelines known as Good Cell Culture Practice (GCCP) [61–64] encourage to realize greater international harmonization and standardization

Table 3
Summary of the results obtained from the MASER-12 suborbital space flight mission (from Tauber et al. 2013 [60]).

Effect	Target molecule									
	CD3	IL-2R	ZAP-70	LAT (pY171)	LAT (pY226)	P-*p*44/42 MAPK	p-tyrosine	Acetyl-histone H3	Vimentin	β-tubulin
Cultivation in experiment hardware (H/W compared to CC)	↓**	–	↓**	↓**	↑**	–	–	↑**	↓**	–
Launch phase / hypergravity (BL compared to H/W)	↓**	↓**	↓*	–	↑*	↓**	↓**	↓**	↓**	–
Microgravity in non-activated T cells (μg and 1g compared to BL)	↓**	↓**	–	–	–	↓*	–	–	–	–
Microgravity in non-activated T cells (direct comparison μg vs. 1g)	–	–	–	–	–	↓*	–	–	–	–
Microgravity in ConA/CD28-activated T cells (μg and 1g compared to BL)	↓*	↓*	–	–	–	–	–	–	–	↑**
Microgravity in ConA/CD28-activated T cells (direct comparison μg vs. 1g)	–	–	–	–	–	–	–	–	–	–

The effect of cultivation in experiment hardware (H/W compared to CC), the effect of launch phase/hypergravity (BL compared to H/W), the effect of microgravity in non-activated T cells (μg and 1*g* compared to BL and direct comparison μg vs. 1*g*) and the effect of microgravity in ConA/CD28-activatedTcells (μg and 1*g* compared to BL and direct comparison μg vs. 1*g*) are demonstrated. (↑*): increase, $p < 0.1$; (↑**): increase, $p < 0.05$ according to two-tailed Mann-Whitney-U-Test. (↓*): decrease, $p < 0.1$, (↓**): decrease, $p < 0.05$ according to two-tailed Mann-Whitney-U-Test. ( ): no significant changes.

Table 4
Summary of observed microgravity effects on human, T lymphocytes cultured *in vitro*.

Category	Effects
Apoptosis	Increase in apoptosis-related factors [21,22]. Increase of DNA fragmentation, PARP protein expression, and p53 and calpain mRNA, increase of 5-LOX activity [23]. Increased p53 phosphorylation [16]. Inhibition of induced programmed cell death [54]. Induction of DNA damage [57].
Cell cycle regulation	Enhanced p21 protein expression, less cdc25C protein expression and enhanced phosphorylation of cyclinB1 [14].
Cell motility	Inhibition of PBMC locomotion [19,20]
Cytokine secretion	Suppressed IL-2 secretion [31,37,40,44] and increased IFNγ secretion [43]. Reduced IFNγ [44].Reduced T cell subsets producing IL-2 and/or IFNγ [45]. Increased IFNγ [35,36] and IL-2 [36] production. Suppressed IFNγ secretion [31].
Cytoskeleton	Structural changes of intermediate filaments of vimentin and of the microtubule network [21,33,34,47].
Epigenetic changes	Differential DNA methylation and chromatin histone modifications [15].
Gene expression	Differential expression of genes involved in DNA repair, cell cycle, cell growth, metabolism, signal transduction, adhesion, transcription, apoptosis, tumor suppression, immune response, cell activation, proliferation and differentiation, protein folding, transport and degradation, cytoskeleton, stress response, apoptosis [14,17,17,42,55–58].
miRNA expression	Altered miRNA expression influencing genes involved in regulation of NF-κB-related signaling network [18].
Mitochondria distribution	Mitochondria clustering and morphological alterations of mitochondrial cristae [47].
PKC distribution	Altered distribution of PKC isoforms [50–52].
Signaling	Hypergravity induced dysregulation in early TCR signaling [60]. Reduced *p*44/42 MAPK phosphorylation [60]. Enhanced MAPK phosphorylation and inhibition of NF-kB translocation [16]. Higher calcium concentration [40]. Activation of fos and NF-kB inhibited [73].
Surface receptor expression	Reduced surface expression of CD25 and CD69 [31,32]. Retarded TCR internalization [32].

of animal and human cell and tissue culture procedures. At the same time, more and more publications are available describing the development of various chemically defined media [65–69]. Due to high standard requirements in production and quality control, industry has already recognized the advantage of fully defined cell culture media and conversion from poorly defined media to chemically defined formulas is set off in many places. This development has led to the commercialization of a growing number of chemically defined media that are available on a regular basis. However, academic research still seems to be disregarding the difficulties and drawbacks arising from the use of sera and other non-defined media supplements, probably due to a deterrent effect of reorganization and not being aware of the benefits clearly outweighing the costs. In fact, as concerns grow regarding global sera supply versus demand [68], prices rise and costs of non-defined and defined media approximate each other. As research in the field of gravitational science is extremely costly and elaborate, resources should be spent deliberately. Therefore, gravitational immunobiology research could profit to a high extent making use of current state-of-the-art for standardization of cell and tissue culture procedures and the development of chemically defined media to achieve an utmost comparability and reliability of results.

The knowledge about the impact of altered gravity conditions on T cell regulation and identification of

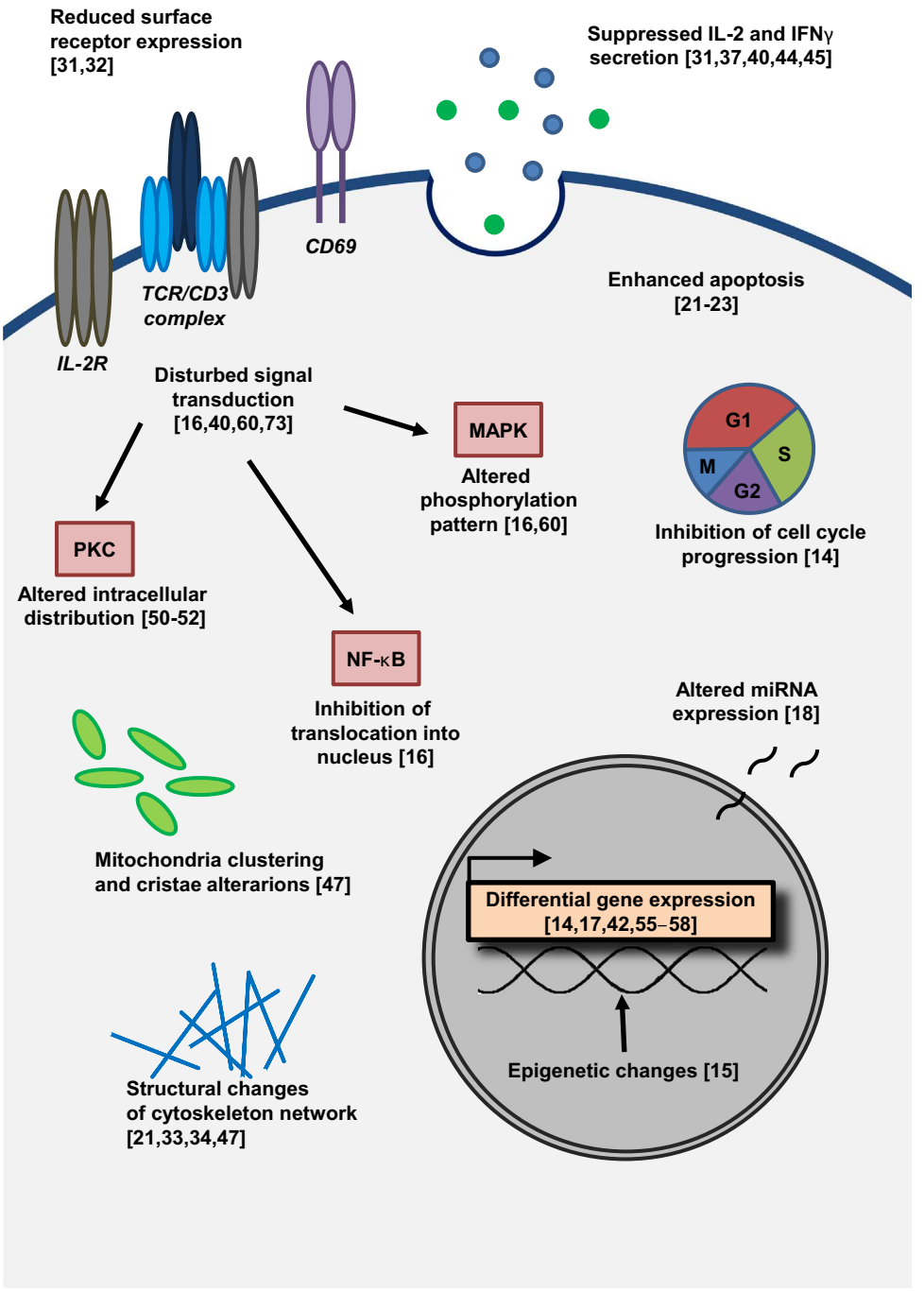

Fig. 1. Schematic summary of the influence of microgravity on T lymphocyte function.

gravity-sensitive cellular reactions will help to understand the molecular mechanisms of disturbed immune cell function in space in order to identify, to test und to provide new targets for therapeutic or preventive intervention related to the immune system of astronauts during long-term space missions [70]. We propose that *in vitro* experiments should follow rigorous standardized cell culture conditions, which are to be developed and agreed by the scientific community.

Acknowledgments

We are also grateful for the financial support from the German Aerospace Center DLR (grant no. 50WB1219). We also express our thanks to Miriam Christen, Sonja Krammer, Josefine Biskup, Jutta Müller, Irina Rau and Marianne Ott. It was only thanks to all these partners that our projects are possible and gratefully acknowledge the contribution of each team member.

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